Final Close-out Report

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PULMONARY-SYSTEM-DISORDERS

Significant Findings.

The results of these studies have demonstrated that cultured primary porcine aortic endothelial cells respond to non-cytotoxic concentrations of asbestos fibers with alterations in specific cell functions, which may be relevant to pulmonary disease processes. Specifically, these data have demonstrated that cultured endothelial cells respond to both chrysotile and crocidolite asbestos fibers by altering cell morphology in focal areas of fiber contact, assuming a "spindled" appearance. This "spindled" phenotype is indicative of the activated endothelial cell; an invasive, pro-inflammatory phenotype which may contribute to inflammation and chronic tissue remodeling known to be a hallmark in asbestos-induced pulmonary disease.

Following endothelial cell exposure to asbestos fibers, this research demonstrated a time-dependent increase in neutrophil (PMN) binding to Intercellular Adhesion Molecule 1 (ICAM-1), and increases in both Urokinase-like Plasminogen Activator (uPA) mRNA levels and uPA activity. Data also revealed that endothelial cells exposed to chrysotile asbestos demonstrated in a time dependent increase in the expression of the cell surface uPA receptor (uPAR).

During the course of this research *in situ* hybridization and zymography techniques were developed to investigate the responses of individual cells to different fibers, thereby providing a means of assessing focal areas of response rather than averaging a response over the entire culture which could include both responding and non-responding cells. *In situ* hybridization and zymography analysis demonstrated that the asbestos-induced increase in uPA mRNA and activity was localized to the activated endothelial cells at sites of fiber deposition.

These studies have also demonstrated that endothelial cells exposed to chrysotile and crocidolite asbestos increase the activation of the transcriptional activating factor Nuclear Factor kappa B (NF-kB), as measured by the nuclear translocation of this complex. Furthermore, this translocation was shown to involve increases in tyrosine phosphorylation and proteolytic activity. This research investigated the nuclear translocation of this *trans*-acting factor as a potential convergence of cell signaling pathways stimulated by asbestos fibers. This demonstrated asbestos-fiber specific translocation may result in the increased expression of the genes shown to be elevated following asbestos fiber exposure in this research.

In contrast, the man-made fiber, Refractory Ceramic Fiber Type 1 (RCF-1) failed to stimulate the endothelial cell morphologic alteration, increased PMN binding, uPA mRNA or activity, or increase NF-kB nuclear translocation.

Taken together, these data suggest that the actions of non-cytolytic concentrations of asbestos are relatively specific, requiring distinct cell signaling pathways. This implies that asbestos fibers act on cells through specific surface and/or chemical interactions rather than by non-specific physical injury. These data also suggest that different fibrous materials, when evaluated at sites of individual cell contact, demonstrate very different cellular reactivity. However, the mechanisms for these discrete cell-specific responses to fibers in endothelial cells remain unclear. These data are intriguing, as they suggest that a pulmonary cell type other than the extensively studied epithelial and mesothelial cells also respond to asbestos fibers and may have role in pulmonary pathophysiology.

Usefulness of Findings.

The focus of these studies was to clarify an individual cell's response to different fibers in order to investigate the mechanisms for altered cell function which may contribute to disease processes *in vivo*. The strength of the cell culture model is that direct effects of fibers can be investigated without the complexity of the effects of additional factors that can be generated by other cell types, such as cytokines or Reactive Oxygen Species (ROS). The direct effects of the fibers demonstrated in these studies were observed at concentrations that did not alter cell viability, therefore these responses do not cause, nor are they secondary to, cell death. Rather, these effects appear to represent activation of the cells to an inflammatory, proliferative, and invasive phenotype. Increases in both ICAM-1 and uPA/uPAR surface expression *in vivo* might explain the vascular remodeling and angiogenesis observed in peripheral regions of the lungs of animals exposed to non-lethal amounts of asbestos. Furthermore, the low concentrations of asbestos required to elicit endothelial cell responses and the fiber-specific nature of these effects suggest that signaling pathways, involved in initiating increased gene expression, are activated by asbestos.

The observation of coordinated expression of ICAM-1 and uPA/uPAR in this research support the hypothesis that asbestos fibers promote endothelial cell activation that is relevant to the pathogenesis of pulmonary fibrosis. Further, the *in situ* methods developed in this research may be translated into *in vivo* inhalation models to demonstrate that the phenotypic changes stimulated in culture is relevant to asbestosis. Developing this connection will provide better understanding of the initiation of fiber-induced disease. The conversion of the cultured cells to a migratory, pro-inflammatory phenotype indicates that these cells could participate in the formation of the highly vascularized granulomanous tissue, which if unresolved, would progress to debilitating pulmonary fibrosis. However, the cell culture model used in these studies is limited. *In vivo* analyses of endothelial cell responses to asbestos fiber deposition following inhalation are needed to fully demonstrate the role of endothelial cells dysfunction in the pathogenesis observed clinically. These studies are very time consuming, costly, and often difficult to adequately control and interpret. Conversely, cell culture models provide more rapid, controlled, cost-effective methods to evaluate individual cell reactivity and fiber toxicity potential.

Because the mechanisms responsible for fiber-induced lung disease remain unclear, it is difficult to know whether the pulmonary disease outcomes demonstrated to date following inhalation of different fiber types can be attributed to a massive fiber burden, rather than the stimulation of discrete cell signaling events at lower fiber doses. This stimulation of discrete cell signaling would be more relevant to occupational exposure potential and disease outcome. Therefore, *in situ* studies like those developed here are beneficial, as they allow the investigation of individual cells to increasing concentrations of varioust fibers, and compare them with the responses of cells to asbestos. Clarifying the responses of relevant pulmonary cell types to different materials in a cell-specific fashion is one practical method to characterize mechanisms of response and to provide a delineation of dose versus mechanistic cellular reactivity. Any results must be confirmed *in vivo*; however, as other contributing cell types and multiple signaling pathways within the lung will ultimately contribute to the larger picture of disease initiation and development in humans.

In order to advance the observations made in this research, a rational approach would be to investigate the *in vivo* relevance of these cell culture model findings. The techniques developed in these studies to date could be used to evaluate whether the pulmonary vasculature demonstrates any of the striking responses of the cultured endothelial cells following asbestos fiber inhalation. *In situ* hybridization protocols developed here could be slightly modified and pulmonary tissue sections from animal inhalation models could be evaluated to determine if the vascular cells of the lung respond *in vivo* with altered expression of uPA mRNA. These sections could also be evaluated histologically for overt signs of neovascularization and endothelial cell activation within the lung. The difficulty in these approaches is to demonstrate that the chronic disease process of asbestosis could be modeled effectively by evaluating the expression of the acute markers of endothelial cell activation detailed in this report. As a result, multiple exposure time points would need to be carefully evaluated.

The immunocytochemical analysis used in this research for detection of uPAR could be exploited to perform immunohistochemical analysis on pulmonary tissue sections to evaluate whether there is an increase in the expression of this receptor following exposure to asbestos fibers in all pulmonary cell types within the section evaluated. The possibility that uPAR is a receptor for asbestos is interesting as a number of cell types, including epithelial cells, express uPAR on their surface, suggesting that uPAR may serve as a global mechanism for pulmonary response to asbestos fibers. Additionally, to investigate the hypothesis that fibers directly interact with uPAR as an initial cell surface event, a column of selected fiber types could be synthesized, over which radiolabeled uPAR could be passed. The interaction of uPAR with different fibers could then be assessed by quantifying either radioactivity in the wash buffer or associated with the column itself. Fibers could be coated with matrix proteins such a vitronectin before investigating uPAR association in this column system as well, in order to clarify the involvement of matrix proteins in the fiber/uPAR association.

The data collected during the course of this funded research, combined with the *in vivo* model analyses and column association experiments described above, could be very important, as many of the structural properties of asbestos are shared with man-made fibers. If it can be confirmed that cell surface receptors such as uPAR are involved in recognition of asbestos, and that this recognition results in cell signaling and altered cell function, techniques such as the column association study could be developed for rapid screening of different fiber type capability to bind uPAR as an indicator of toxicity potential. A global implication of the observations made during the term of the R03 is that various fiber types may differ in their cellular interactions and resulting cell reactivity, thereby making their potential to initiate fibrotic diseases quite different.

The restriction of asbestos fiber use in industrial applications has established the need for other fibrous materials with favorable durability and heat resistance to be expanded. This expansion demands that significant research effort be focused on determining the fibrogenic or carcinogenic potential of these man-made fibers. A rapid *in vitro* screening approach to ascertain recognition of a fiber for a receptor such as uPAR would be inexpensive and reproducible.

Abstract.

Asbestosis is a lung disease associated with increased fibrinoloysis, expansion of interstitial matrix components, and angiogenesis, all of which have been associated with activated endothelial cells. However, the cellular or molecular effects of asbestos or other fibers on the vascular cells are unknown. The purpose of this research was to investigate the specific hypothesis that asbestos exposure induces an activated endothelial cell phenotype, resulting in increased transcriptional activation and expression of gene products relevant to pulmonary dysfunction. Non-cytolytic concentrations of chrysotile and crocidolite asbestos, but not a ceramic fiber, non-asbestos containing RCF-1, caused the development of a localized "spindled" morphology consistent with an activated phenotype in primary cultures of porcine aortic endothelial cells. This activated sub-population of endothelial cell cultures was principally responsible for a time-dependent chrysotile and crocidolite asbestos-induced 2 to 4 fold increase in neutrophil adherence to ICAM-1 as determined by fluorescent and myeloperoxidase enzyme activity adherence assays and monoclonal antibody blocking experiments. There was a 2 to 7 fold increase in steady state uPA message as determined by solution hybridization, which was demonstrated to be localized to activated endothelial cells, using *in situ* hybridization techniques. There was also a demonstrated, localized increase in the activity of this serine protease as detected by *in situ* zymography. In addition, electrophoretic mobility shift assays have demonstrated that chrysotile and crocidolite asbestos, but not RCF-1, induce a tyrosine phosphorylation and protein degradation-dependent increase in nuclear translocation of the trans-acting factor NF-kB. Taken together, these data demonstrate that the endothelial cells respond to chrysotile and crocidolite asbestos, but not RCF-1 fibers by assuming an activated phenotype, altering transcriptional activation, and expressing gene products relevant to pulmonary tissue remodeling. It is postulated that similar events in lung endothelial cells *in vivo* may contribute to asbestos-induced lung disease in man.

Final Performance Report.

The purpose of the funded research was to investigate the specific hypothesis **that asbestos induces an active endothelial cell phenotype, resulting in the increased expression of growth factors and proteases, which are relevant to the development of fibrosis**. The specific aims of this research project were to:

1. Optimize *in situ* techniques to investigate altered steady state messenger ribonucleic acid (mRNA) levels and protein synthesis following exposure of endothelial cells to fibrogenic substances.
2. Demonstrate that asbestos alters synthesis and release of active Basic Fibroblast Growth Factor (bFGF), uPA, and Transforming Growth Factor Beta 1 (TGF-B1).
3. Compare various man made fibers for their potential to cause endothelial cell activation.

As detailed in the first progress report filed on June 28, 1995, specific aim #2 was focused to demonstrate that asbestos alters the synthesis and release of uPA. Studies were narrowed to focus on uPA due to its central role in endothelial cell activation, the interesting results achieved during the first several months of the research, and given the complexity of the control of uPA, it was determined that diluting the research effort to investigate bFGF and TGF-B1 as well during this two year period was beyond the attainable scope of this project.

The background for the project, procedures, methodology, results and discussion, and conclusions are fully and most appropriately summarized in the thesis text entitled, "Activation of vascular endothelial cells in response to mineral fibers" included with this report.

Publications.

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